VARYING HEMOLYTIC AND CONSTANT COMBINING CAPACITY OF STREPTOLYSINS; INFLUENCE ON TESTING FOR ANTISTREPTOLYSINS

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While investigating the antistreptolysin content of patients' sera according to Todd's method (1–3), we found that the hemolytic titer of one given streptolysin might vary considerably within a short period. The purposes of this communication are: first, to show that although the hemolytic titer of such a streptolysin does vary, its power of combining with antistreptolysin is constant under certain conditions; and second, to discuss the effect of this phenomenon upon the standardization of streptolysins which are to be used in testing sera for antistreptolysins.

Neill and Mallory (4) demonstrated that streptolysin may exist in at least two forms: (*a*) reduced and hemolytic, and (*b*) oxidized and non-hemolytic. They further showed that these two forms are easily reversible, one to the other, by oxidation and reduction. Such a reversibility would readily explain variations in hemolytic titer because the degree of hemolysis depends upon the amount of the reduced (hemolytic) form alone; such a reversibility, nevertheless, might not change the total streptolysin present in a given lot of hemolytic broth. It seemed to us that this hypothesis predicates a constant combining capacity of streptolysin with antistreptolysin even though the hemolytic strength might vary. In order to test it the following experiments were performed.

Large amounts of streptolysins Nos. 32, 33, and 35 were made. Each batch was divided into several different samples and each sample was subjected to different conditions, in order to obtain different hemolytic titers. In these experiments, the amount of hemolytic broth required to hemolyze completely 0.5 cc. of 5 per cent red blood cells in 1 hour was determined; this is known as 1 M.H.D. (minimal

hemolytic dose) (1). Simultaneously, the amount of serum required to neutralize 0.5 cc. of hemolytic broth was determined. The results are shown in Table I.

In Table I it is seen that the amount of a given hemolytic broth required to hemolyze completely 0.5 cc. of 5 per cent red blood cells varied within wide limits, while, on the other hand, 0.5 cc. of one lot

TABLE I

Constant Combining Capacity Compared with Varying Hemolytic Strength of Three Different Lots of Streptolysin

Streptolysin No.	Conditions to which streptolysin was subjected	Amount of hemolytic broth required to hemolyze 0.5 cc. of 5 per cent red blood cells	No. of M.H.D. per 0.5 cc. of hemolytic broth	Amount of serum required to neutralize 0.5 cc. of hemolytic broth
		cc.		*cc.*
32	A. Freshly opened, prepared, and reduced 2 wks. previously	0.18	2.8	1/120 serum Eis
32	B. A exposed to room temperature and air 8 hrs.	0.04	12.5	1/120 " "
32	C. Freshly opened, reduced 2 days previously	>0.40	<1.2	1/120 " "
32	D. 1 part A, 3 parts unreduced	0.18	2.8	1/120 " "
32	E. Reduced day of using, exposed to air and room temperature 8 hrs.	0.06	8.3	1/120 " "
33	A. Freshly opened, prepared, and reduced 1 wk. previously	0.20	2.5	1/250 " "
33	B. A after 8 hrs. unsealed at room temperature and 16 hrs. unsealed in the ice box	0.08	6.2	1/250 " "
35	A. Immediately after being made and reduced	0.14	3.6	1/100 " Val
35	B. A after 24 hrs. in the ice box	0.08	6.2	1/100 " "

of streptolysin was always neutralized by a constant amount of one serum. Thus, in the case of streptolysin No. 32, 0.04 cc. of Sample B completely hemolyzed 0.5 cc. of 5 per cent red blood cells, though 0.40 cc. of Sample C was insufficient to accomplish this. Nevertheless, 0.5 cc. of both Samples B and C required 1/120 cc. of serum for neutralization; hence, the hypothesis above outlined was substantiated.

The constancy of combining power compared with the variability of hemolytic power made it desirable to determine whether a streptolysin, completely non-hemolytic because in the unreduced state, still had the same combining capacity as when it was reduced. By setting up an experiment in stages, it was demonstrated that unreduced (non-hemolytic) streptolysin No. 32 had practically the same combining capacity as is shown in Table I.

Repeated experiences of this type made it obvious that one must find a method for standardizing streptolysin other than that originally

TABLE II

Influence of Both Varying and Keeping Constant the Dose of Hemolytic Broth

Streptolysin No. 32	Condition of streptolysin	Amount of hemolytic broth producing complete hemolysis	Amount of hemolytic broth used in test	No. of M.H.D.	Amount of serum required for neutralization
		cc.	*cc.*		*cc.*
A. Using 2½ M.H.D. as a constant	Immediately after reduction	0.2	0.5	2½	1/250
	1 hr. after reduction	0.1	0.25	2½	1/444
	24 hrs. after reduction, kept cold	0.04	0.1	2½	1/1,200
B. Using constant amounts of hemolytic broth regardless of hemolytic titer	Immediately after reduction	0.2	0.5	2½	1/250
	1 hr. after reduction	0.1	0.5	5	1/250
	24 hrs. after reduction, kept cold	0.04	0.5	12½	1/250

recommended by Todd (1), and which he later found it necessary to modify. Theoretically 1 streptolysin unit would be neutralized by 1 antistreptolysin unit. Todd (1, 2) defined 1 antistreptolysin unit in a given serum as the amount that would neutralize 2½ minimal hemolytic doses of streptolysin when 1 minimal hemolytic dose (M.H.D.) would completely hemolyze 0.5 cc. of 5 per cent rabbit red blood cells. According to this definition 1 combining unit of streptolysin should correspond to 2½ hemolytic doses. That such a correspondence does not constantly exist is shown in Table I and is even better illustrated in the following experiment.

Streptolysin No. 32 was reduced *in vacuo* with 0.1 per cent sodium hydrosulfite. Portions of this streptolysin kept in the ice box during the period of the experiment were tested simultaneously for hemolytic and combining power at the following periods: immediately after reduction, 1 hour, and 24 hours later. The results are shown in Table II.

Table II illustrates the unreliability of standardizing the combining capacity of streptolysins by their hemolytic strength. It further shows the necessity of discarding the $2\frac{1}{2}$ M.H.D. standard and adopting one based on the total streptolysin content of a given reagent. If attempts had been made to standardize this lot of streptolysin by the $2\frac{1}{2}$ M.H.D. method, its combining capacity would have apparently varied almost 500 per cent. When, on the other hand, the amount of streptolysin-containing broth was kept constant in spite of a varying hemolytic titer, its combining capacity was found to be constant.

Todd (1) stated that it was impossible to titrate streptolysins accurately by their hemolytic capacity because of technical difficulties in determining their hemolytic strength, and also because non-hemolytic streptolysin had some power of combining with antistreptolysin. To test the latter point he used streptolysin so altered by long exposure to air that it could not be reduced to the hemolytic form. This altered streptolysin had lost all of its hemolytic power and well over half of its combining capacity. These changes were apparently irreversible (1, 5). In contrast to this, our experiments demonstrate still another reason for titrating streptolysins according to their combining capacity; namely, the easy and rapid reversibility of the non-hemolytic to the hemolytic form.

It will also be observed from Table II that reduction progressed over a period of 24 hours. Such an observation suggested that it might be possible to push reduction to a point of completion, where all of the unreduced (non-hemolytic) form had been converted into the reduced (hemolytic) form. In such a condition, there should be a rough parallelism, at least, between the hemolytic titer and combining capacity of all streptolysins thus treated. We have, however, been unable to obtain any such parallelism with regularity, possibly because of the presence of varying amounts of streptolysin so altered that it could no longer be reduced to the hemolytic form, although it still retained some power of combining with antibody.

Combining Stability of Streptolysin

All of the data so far presented, suggested that it might be possible to prepare and reduce a large amount of streptolysin at one time, standardize it with a number of sera of known antistreptolysin strength,

TABLE III

Comparative Constancy of Combining Power of Various Lots of Streptolysin

Streptolysin No.	Date of preparation and reduction	Date of test	Amount of serum required to neutralize 0.5 cc. of streptolysin	Serum
			cc.	
26	Aug. 26, 1932	Oct. 28, 1932	1/160	Wei
26	" 26, 1932	May 13, 1933	1/133	"
26	" 26, 1932	Oct. 20, 1932	1/111	Sch
26	" 26, 1932	May 13, 1933	1/100	"
26	" 26, 1932	Oct. 28, 1932	1/40	McE
26	" 26, 1932	May 13, 1933	1/50	"
33	Jan. 25, 1933	Feb. 5, 1933	1/62	Br
33	" 25, 1933	May 14, 1933	1/62	"
33	" 25, 1933	Jan. 26, 1933	1/300	Eis
33	" 25, 1933	Feb. 1, 1933	1/260–1/300	"
33	" 25, 1933	Feb. 5, 1933	1/260	"
33	" 25, 1933	May 14, 1933	1/300	"
33	" 25, 1933	Feb. 1, 1933	1/250	Val
33	" 25, 1933	Feb. 3, 1933	1/240	"
33	" 25, 1933	Feb. 5, 1933	1/225	"
33	" 25, 1933	Feb. 6, 1933	1/225	"
33	" 25, 1933	May 14, 1933	1/240	"

and use it as a standard reagent over a considerable period. The stability of such a reagent is illustrated in Table III.

The variations noted in Table III are within the limits of error of the method employed.

In order to maintain this stability it has been necessary to keep the flasks containing the reduced streptolysin in the refrigerator and well sealed with vaseline. Certain lots not sealed, even though kept in the cold, have become weaker in both hemolytic and combining

strength; and while this has not been a constant phenomenon, it has occurred often enough to make advisable the use of freshly opened streptolysin.

To determine further the effect of exposure to air a number of flasks of the reagent were opened, part of the contents removed, and the flasks immediately sealed and replaced in the ice box. Tests made at the time of original opening and later are presented in Table IV.

The results in Table IV indicate that reduced streptolysin opened and resealed may be safely used within a few days, but that less reliance may be placed on such reagents kept longer periods.

TABLE IV

Constancy of Combining Power of Streptolysin Opened and Resealed

Streptolysin No.	Serum	Amount of serum required to neutralize 0.5 cc. of streptolysin at time of original opening and resealing	Interval between tests	Amount of serum required at time of second testing
		cc.		*cc.*
33	Wer	1/180	3 mos.	1/200
34	Ste	1/33	1 mo.	1/30
34	"	1/33	2 days	1/33
34	N-99	1/100	1 mo.	1/100
34	Wel	1/140	1 day	1/125
34	"	1/140	2 days	1/140
34	Rap	1/140	2 "	1/140
35	Wel	1/166	3 mos.	1/200

DISCUSSION

The data here presented indicate that for the purpose of determining the antistreptolysin content of a given serum it is essential to know the combining capacity of the streptolysin used rather than to determine its exact hemolytic power in terms of minimal hemolytic dosage.

The technical difficulties in the actual test arise from the fact that streptolysin plays at least two different rôles *in vitro*: first, that of a substance which combines with antibody, apparently in multiple proportions, in the first stage of the reaction, and second, that of an indicator which makes itself manifest by lyzing erythrocytes in the second stage. The streptolysin exerts the first function either in the

oxidized or reduced state, and the second function only in the reduced form. To titrate the exact point where streptolysin and antistreptolysin have completely combined one with the other, it is necessary to have additional tubes containing some uncombined, reduced (hemolytic) streptolysin which will lyze the erythrocytes at all points in excess of that where the total streptolysin has been rendered completely inert by the antibody. Any excess amount of free active lysin will indicate where this point lies; hence the last tube where no hemolysis occurs shows where the union of antigen (streptolysin) and antibody has been complete. The important factor is that this streptolysin must be free in order to attack the erythrocytes and active in order to lyze them.

Originally Todd (1, 2) produced streptolysin by means of growing the streptococci anaerobically in a yeast extract reinforced broth sterilized by filtering through a Chamberland candle. The reagent thus produced seemed to contain about $2\frac{1}{2}$ M.H.D. per 0.5 cc. of broth and to possess equivalent hemolytic and combining powers. The same strain of streptococcus, however, when grown aerobically in broth reinforced with dextrose, certain buffers, and salts (6, 7) has yielded much stronger concentrations of streptolysin per cubic centimeter of medium. Reagents produced in the latter way have not shown any constant parallelism between hemolytic and combining powers. The influence of varying degrees of oxidation and reduction in this media, with corresponding variations in amounts of hemolytic, compared with non-hemolytic streptolysin, has made it necessary to change the methods of standardization of the reagent. If precise and constantly reproducible conditions for complete reduction of streptolysin to an actively hemolytic form could be determined, the standardization of streptolysin in terms of $2\frac{1}{2}$ M.H.D. would be possible. Up to the present time, however, we have been unable accurately to define these conditions.

The fact, on the other hand, that a given amount of streptolysin kept under certain conditions has a constant combining capacity which is doubtless fixed regardless of whether it is in the reduced or oxidized form, or in a combination of the two, makes the standardization of a given lot of streptolysin more simple. The additional fact that the reagent maintains its combining capacity at a fairly constant

level, provided it is kept sealed and cold, makes easier the preservation of a quantity of standardized reagent.

Todd (8) has suggested that a serum containing a known number of antistreptolysin units be universally employed as a standard;[1] but up to the present no such standard serum has been available. In order to keep the same system of units already employed by Todd, we have standardized our streptolysin with a number of sera furnished by Dr. Coburn, as described under Technique 4; duplicates of these sera had been tested by Todd. About 10 per cent of them showed wide variation from the results obtained by Todd, who (8) has suggested to us that the discrepancies are due to contamination of the sera; for as a rule we have found that sera kept sterile and cold have maintained quite constant their antistreptolysin content.

After the completion of this work Todd's latest paper (5) appeared showing that both reduced and unreduced streptolysins are active antigens *in vivo*, in that they both induce the production of antistreptolysin when injected into animals; and incidentally he showed both here and in his original paper (1) that irreversibly oxidized streptolysin combined with antistreptolysin, though in different proportions than did reduced streptolysin. Furthermore, streptolysin produced in serum broth, while acting as an antigen *in vivo* would not combine with antistreptolysin *in vitro*, even though it would lyze erythrocytes. This work furnishes additional proof of the necessity of determining accurately the dosage and conditions under which a streptolysin will combine with antistreptolysin if it is to be used for measuring the amount of the antibody in a given serum.

SUMMARY

Certain properties of streptolysin in respect of hemolytic power and capacity for combining with antistreptolysins have been determined. The hemolytic strength may vary markedly, while under suitable conditions the combining power is constant.

This stability of combining capacity makes it possible to prepare a

[1] By antistreptolysin units Todd means the reciprocal of the fraction of a cubic centimeter of serum that will neutralize a standard amount of streptolysin. Obviously this amount of streptolysin must have been previously standardized according to the method herein described.

large amount of streptolysin, standardize it against sera of known antistreptolysin strength, and use it as a reagent over a considerable period for testing the antistreptolysin content of unknown sera. A modified technique for making these tests is described.

APPENDIX

Technique

So many modifications of Todd's original technique (1) have been introduced that the following steps are described.

1. Making the Broth (6, 7).—Fresh beef heart is freed of fat, finely ground, mixed with tap water in the proportion of 1 pound per liter, infused 16 hours in the ice box, heated slowly to 85°C., where it is maintained for 30 minutes. It is filtered through Pratt-Dumas filter paper, the meaty pulp squeezed dry, and 20 gm. of Difco proteose peptone dissolved in each liter of infusion. The reaction is adjusted to pH 8; the broth is then filtered through No. 12 Whatman filter paper, and placed in flasks. It is sterilized in the Arnold apparatus on 3 successive days. Each liter is then reinforced with the following solution which is sterilized by filtering through a Berkefeld candle before addition to the meat infusion: dextrose 2 gm., $NaHCO_3$ 2 gm., NaCl 2 gm., $Na_2HPO_4 \cdot 12\ H_2O$ 1 gm., water 50 cc. The reinforced broth should be incubated to insure sterility.

2. Production of Streptolysin.—The broth, warmed to 37°C., is heavily inoculated with an actively growing culture of a suitable hemolytic streptococcus[2] incubated for 16 hours—then filtered through a Chamberland F candle, with the precaution of discarding the first 100 cc. of filtrate.

This filtrate is then reduced *in vacuo* by adding 1 gm. of freshly ground sodium hydrosulfite to each liter, and maintaining a high vacuum until only a few bubbles are given off. The addition of NaOH as originally advised by Todd has usually been found superfluous, as the pH of the streptolysin after reduction is about 7.4 to 7.8. The essential requirement is that the final reaction after complete reduction remain slightly alkaline. The streptolysin is placed in tubes or small flasks and sealed with a layer of warm (not hot) sterile vaseline, about 5 cm. thick, and stored in the ice box.

3. Titration of Hemolytic Power.—Beginning with 0.5 cc. of hemolytic broth, decreasing quantities in steps of about 10 per cent are placed in a series of Wassermann tubes. Physiological saline is added to make the total volume in each tube equal 1.5 cc. Then 0.5 cc. of 5 per cent washed red blood cells (rabbit) is added, quickly and thoroughly shaken, and incubated 1 hour. The last tube showing complete hemolysis contains 1 M.H.D.

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[2] We have employed Strain W P R L furnished by Dr. Todd.

4. Determining the Standard Combining Power.—In a series of tubes the streptolysin is diluted by 10 per cent decrements; the volume in each is made 0.5 cc. with physiological salt solution, then 1 cc. dilution of a known standard serum is added, the serum having been diluted so that 1 cc. equals 1 unit. The mixture is shaken immediately and after 15 minutes, is incubated 1 hour, when readings are made. The last tube showing no hemolysis represents the point of complete neutralization, or in other words, 1 combining unit. This testing should be done with several sera of known unit value, high, low, and medium, before the standard dose of a given streptolysin is fixed.

5. Testing Human Sera for Antistreptolysin.—Todd's technique (2) calls for a preliminary testing with 1:10, 1:100, and 1:1,000 dilutions of the serum and a

Protocol 1

Scheme for Diluting Serum to Obtain a Given Number of Units of Antistreptolysin per Tube

Normal salt solution	0.0	0.5							
Serum 1:25 dilution	1.0	0.5							
Tube represents antistreptolysin units	25	50							
Normal salt solution		0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Serum 1:100 dilution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cc. employed in test	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Tube represents antistreptolysin units	100	150	200	250	300	350	400	450	500
Cc. employed in test					0.5	0.5	0.5	0.5	0.5
Tube represents antistreptolysin units					600	700	800	900	1,000
Cc. employed in test					0.25	0.25	0.25	0.25	0.25
Tube represents antistreptolysin units					1,200	1,400	1,600	1,800	2,000

second titration using 0.1 cc. increments of the dilution indicated by the preliminary test. Measured in units this gives a very uneven gradation. We have, therefore, adopted a system whereby the gradations consist of 50 units from 50 to 500, 100 units from 500 to 1,000, and 200 units from 1,000 to 2,000. As the majority of sera contain less than 500 units, this is the usual limit; and a second testing with the higher dilutions is only occasionally necessary. Where the approximate range is known still fewer tubes are required.

Two dilutions of serum to be tested, 1:25 and 1:100 respectively, are made with physiological salt solution, and subsequently diluted as shown in Protocol 1.

Where necessary the volume of dilution in each tube is made up to 1 cc. with physiological salt solution. The previously standardized streptolysin is diluted with physiological salt solution so that 0.5 cc. represents 1 combining unit as pre-

viously determined in Step 4. This amount is added to each tube of diluted serum, well mixed and incubated in the water bath at 37°C. for 15 minutes, then 0.5 cc. of 5 per cent washed red blood cells (rabbit) is added, well shaken immediately and again after 15 minutes' incubation. The tubes are incubated for a total of 60 minutes, and the readings made immediately. The last tube showing no hemolysis represents the number of units of antistreptolysin in the serum tested. Control series containing no serum, serum of known low titer, and serum of known higher titer are introduced each day; thus a constant check on the combining capacity of the streptolysin is maintained; and when it varies appreciably from that originally determined the tests for the day are discarded.

Todd (8) has suggested to us that the streptolysin be diluted with broth similar to that in which it was originally made; but we have found that some lots of broth apparently inactivate the streptolysin. Physiological salt solution, on the other hand, has proven completely indifferent. It is advisable to dilute only approximately the amount of streptolysin necessary for 1 day's work, because of the danger of deterioration after dilution. A new lot of streptolysin may be conveniently standardized by running parallel tests with the new and with the standard streptolysin with various sera at the time the antistreptolysin contents of these sera are determined.

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